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Neuropilin-1 modulates poly(ADP-ribose)-polymerase expression leading to reduced endothelial apoptosis in cerebral ischemia

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2 Summary

Vascular Endothelial Growth Factor(VEGF) inhibits endothelial apoptosis through upregulation of Poly(ADP-ribose)-Polymerase(PARP) expression. Neuropilin-1(NP-1) is an alternative receptor for VEGF and possibly a fine-regulator of the endothelial response to ischemia. In the cerebral vasculature the mechanisms of apoptosis induction and inhibition are not fully understood. This study investigated the impact of NP-1 on endothelial PARP expression and its modulatory role in apoptosis inhibition by VEGF.

VEGF treated endothelial cell cultures showed NP-1 and VEGF-R2 complexing and enhanced PARP expression. An inhibition of VEGF/VEGFR-2 interaction on apoptosis induced by adhesion inhibition through the αV-integrin inhibitor cRGD was augmented. Cerebral ischemia and endothelial apoptosis were investigated by MRI and immunohistochemistry in rat models of permanent and temporary middle cerebral artery occlusion. Ischemic hemispheres displayed apoptosis and increased endothelial NP-1 and VEGFR-2 expression compared to non-ischemic hemispheres and sham-operated or untreated controls. Increased SOD-1 and Catalase expression indicated oxidative stress in the ischemic brain. Endothelial PARP expression remained stable during cerebral ischemia despite pro-apoptotic conditions.

NP-1 upregulation reinforced apoptosis inhibition and modulated VEGF-dependent PARP.

Keywords: Vascular Endothelial Growth Factor Receptor, Neuropilin-1, apoptosis,cerebrovascular system, rat middle cerebral artery occlusion (MCAO) model

3 Zusammenfassung

Der vaskuläre endotheliale Wachstumsfaktor VEGF hemmmt die Endothelzellapoptose über eine Hochregulierung von Poly(ADP-ribose)-Polymerase(PARP). Neuropilin-1(NP-1) ist ein alternativer Rezeptor für VEGF und möglicherweise ein Feinregulator der Endothelantwort bei zerebraler Ischämie. Der Mechanismus der Apoptoseinduktion und –hemmung in zerebralen Gefäßen ist noch ungeklärt. Diese Studie untersucht den Einfluss von NP-1 auf die endotheliale PARP Expression und seine Rolle bei Apoptoseinhibierung durch VEGF.
Mit VEGF behandelte Endothelzellkulturen zeigten Komplexierung von NP-1 und VEGFR-2 sowie erhöhte PARP-Expression. Eine Hemmung der VEGF/VEGF-R2 Interaktion bei Apoptose wurde beobachtet. Zerebrale Ischämie und endotheliale Apoptose wurden anhand eines Rattenmodels durch temporäre und permanente Okklusion der A.cerebri media mittels MRT und Immunhistochemie untersucht. Ischämische Hemisphären wiesen neben Apoptose erhöhte NP-1 und VEGFR-2 Expression im Vergleich zu nicht ischämischen Hemisphären und Sham-operierten Tieren oder unbehandelten Kontrollen auf.
Erhöhte SOD-1 und Catalase-Expression indizierten oxidativen Stress bei Ischämie. Die endotheliale PARP Expression blieb während Ischämie trotz proapoptotischer Bedingungen stabil.
Die Hochregulierung von NP-1 verstärkt die Apoptosehemmung und moduliert VEGF-abhängig die PARP-Expression.

Schlüsselwörter: Vascular Endothelial Growth Factor Receptor, Neuropilin-1, Apoptose, zerebrovaskuläres System, Mittlere-Cerebrale-Arterie-Okklusions (MCAO)-Modell

4 Article and authors

Neuropilin-1 modulates poly(ADP-ribose)-polymerase expression leading to reduced endothelial apoptosis in cerebral ischemia

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5 Abstract

Cerebral ischemia is encompassed by vascular apoptosis leading to cerebrovascular damage, yet the mechanisms of apoptosis induction and inhibition in the cerebral vasculature are not fully understood. We have previously demonstrated inhibition of endothelial apoptosis by vascular endothelial growth factor through upregulation of poly(ADP-ribose)-polymerase expression. Here, we hypothesized that neuropilin-1, an alternative receptor for vascular endothelial growth factor, is a fine-regulator of the endothelial response to cerebral ischemia. This study investigated the impact of neuropilin-1 on endothelial poly(ADP-ribose)-polymerase expression and in turn, its modulatory role in apoptosis inhibition by vascular endothelial growth factor in the cerebrovascular system.

In endothelial cell cultures, neuropilin-1 acted as a co-receptor to vascular endothelial growth factor receptor-2. This enhanced poly(ADP-ribose)-polymerase mRNA and protein expression induced by vascular endothelial growth factor-A, as demonstrated

by incubations with receptor-specific inhibitors and vascular endothelial growth factor isoforms. Neuropilin-1 colocalized with vascular endothelial growth factor receptor-2 and augmented the inhibitory effect of vascular endothelial growth factor / vascular endothelial growth factor receptor-2 interaction on apoptosis that was induced by adhesion inhibition through the αv-integrin inhibitor cRGDfV. Neuropilin-1 signal transduction involved c-Jun N-terminal kinase and Akt. Cerebral ischemia and endothelial apoptosis were investigated in rat models of permanent and temporary middle cerebral artery occlusion. Animals were evaluated neurologically and by cerebral magnetic resonance imaging for edema formation after 24 hours. In brains analyzed by in situ-ligation apoptosis assay and immunohistochemistry, vessels of ischemic cerebral hemispheres displayed apoptosis and increased endothelial neuropilin-1 and vascular endothelial growth factor receptor-2 expression compared to non-ischemic cerebral hemispheres and sham-operated or untreated controls. Increased vascular superoxide dismutase-1 and catalase expression indicated oxidative stress in the ischemic brain. Of note, endothelial poly(ADP-ribose)-polymerase expression remained stable during cerebral ischemia despite pro-apoptotic conditions.

Neuropilin-1 is upregulated in conditions of imminent endothelial apoptosis to reinforce apoptosis inhibition and to modulate vascular endothelial growth factor-dependent poly(ADP-ribose)-polymerase expression in the cerebral vasculature. We propose that neuropilin-1 is a key modulator of vascular endothelial growth factor maintaining endothelial integrity during ischemia. Modulating the function of neuropilin-1 to target poly(ADP-ribose)-polymerase expression could help to prevent endothelial apoptosis in the treatment of cerebral ischemia.

6 Introduction

Neuropilin-1 (NP-1) is an alternative receptor for vascular endothelial growth factor (VEGF) (Soker et al., 1998). NP-1 has been described to function as a coreceptor to VEGF receptor-2 (VEGFR-2), the main receptor for VEGF on vascular endothelial cells (EC) (Zachary, 2001; Neufeld et al., 1999). NP-1 potentiates the effects of VEGF-A(165) binding to VEGFR-2 by increasing the cellular response to VEGF (Soker et al., 1998) and by generating a stronger VEGFR-2 signal (Ballmer-Hofer et al., 2011). VEGF is the strongest apoptosis-inhibiting factor of EC supporting endothelial survival and regeneration of vascular integrity. Among the different members of the VEGF family, VEGF-A is the most important for the vascular system with several splice variants (121, 145, 165, 189, and 206 amino acids) that show different receptor affinities. VEGF-A(165) shows the strongest biological activity on EC promoting survival, proliferation and migration, but also vascular permeability (Zachary, 2001). In the cerebral vascular system, arterial occlusion leads to endothelial apoptosis (Li et al., 1995), followed by the breakdown of the blood brain barrier and cerebral edema formation, as previously demonstrated (Gerriets et al., 2004 a; Gerriets et al., 2004 b).

Endothelial apoptosis is a characteristic result not only of cerebral ischemic disease, but also of atherosclerosis or autoimmune vasculitis (Choy et al., 2001; Winn and Harlan, 2005). Endothelial apoptosis and survival are subject to complex regulation by pro- and anti-apoptotic factors, the most important anti-apoptotic being VEGF. EC survival is governed by growth factors and also by adhesion molecules such as

integrins (Choy et al., 2001). We have previously demonstrated apoptosis induction through the inhibition of vitronectin-dependent adhesion of endothelial and vascular smooth muscle cells with subsequent activation of caspase-3 (Al-Fakhri et al., 2003 a). Caspases are involved in the transduction of apoptosis-inducing signals to the nucleus. Caspase-3 induces typical apoptotic features such as DNA degradation by endonucleases or inhibition of DNA repair through cleavage of poly(ADP-ribose)-polymerase (PARP) (Rossi and Gaidano, 2003). PARP is a nuclear DNA-binding enzyme that repairs DNA strand breaks and counteracts pro-apoptotic deoxyribonucleases. It is inactivated by active caspase-3 at an early stage of the apoptotic process (Duriez and Shah, 1997). In a previous study, we reported that VEGF specifically induced PARP expression and activity thereby inhibiting EC apoptosis (Hörmann et al., 2011). PARP is also involved in inflammation and oxidative stress (Virag, 2005; Aguilar-Quesada et al., 2007) by modulating the activity of several transcription factors as promoter binding cofactor, like p53, NF-κB, and STATs. The role of PARP in cell death and inflammatory responses depends on the cellular activation state. In oxidative stress situations with excessive and unrepairable DNA damage, such as acute inflammation, shock, or severe ischemia, overactivation of PARP leads to intracellular ATP depletion resulting in necrosis. In the absence of overactivation, PARP conveys cellular protection (Virag, 2005; Aguilar-Quesada et al., 2007).

There are several indications of a modulatory role for NP-1 in VEGF/VEGFR-2 interactions. However, the intracellular mechanisms by which NP-1 exerts its effect on the endothelial VEGF response remain to be identified. We hypothesized that NP-1 modulates PARP expression that is induced by VEGF through VEGFR-2

signaling and thereby adapts the cellular response to VEGF. NP-1 could act as a protective vascular receptor involved in VEGF-dependent processes such as apoptosis inhibition in cerebral ischemia. In this study, we investigated the role of NP-1 in the inhibition of endothelial apoptosis by VEGF and the effect on PARP regulation in cerebral ischemia. We show that NP-1 is an important modulator of VEGF effects through the regulation of PARP expression influencing endothelial survival during cerebral insults.

7 Materials and methods

7.1 Cell cultures

Human umbilical vein EC (HUVEC) (PromoCell) and the macrovascular EC line EA.hy.926 (Edgell et al., 1983) were cultivated for 2 days (d) to 60-70% confluence with endothelial growth medium without VEGF supplement (Promocell), thereafter incubated as follows:

7.2 Incubation protocols

HUVEC and EA.hy.926 were incubated with recombinant human homo-dimeric VEGF-A(165) or VEGF-A(121) 1-100ng/ml ($5x10^{-11}$ - $5x10^{-9}$M) (Peprotech) over 16 hours (h) to 24h for mRNA isolation, over 20h for apoptosis detection, or over 24h to 6d (with repeated incubations at intervals of 48h) for protein isolation, respectively.

After stimulation with VEGF to increase the apoptotic threshold, as previously described (Hörmann et al., 2011), apoptosis was induced by incubation with αV-integrin inhibitor cRGDfV (cyclo-[Arg-Gly-Asp-D-Phe-Val]) 5µg/ml (Bachem) for 24h

on vitronectin (2µg/ml)-coated multiwell plates, blocked with 3% BSA (Al-Fakhri et al., 2003 a).

To analyze the function of NP-1 and VEGFR-2, the above mentioned cells were incubated with VEGF-A(165) 10ng/ml in the presence or absence of the peptide inhibitor of NP-1 A7R [ATWLPPR] (Bachem) 10µM and/or the selective VEGFR-2 tyrosine-kinase inhibitor SU5416 [3-[(2,4-dimethylpyrrol-5-yl)-methylidene]-indolin-2-one] (Calbiochem) 10µM in the presence or absence of cRGDfV 5µg/ml.

VEGF signal transduction was studied by VEGF-A(165) 100pg/ml-100ng/ml incubation over short time intervals (15-30-60-120min). Involvement of Akt and JNK was analyzed by incubation with Akt inhibitors triciribine 10µM, isozyme-selective Akti-1/2 1µM or SAPK/JNK inhibitor SP600125 10µM (Calbiochem), respectively, and VEGF-A(165) 1ng/ml-100ng/ml.

Controls were conducted in parallel to all experiments using only EC medium (negative control), only VEGF-A(165) 10-100ng/ml (VEGF control) or cRGDfV (apoptosis control).

7.3 Animals and MCAO model

Ischemia was assessed using a permanent and a temporary middle cerebral artery occlusion (MCAO) model, as described previously (Gerriets et al., 2004 b). All procedures were in accordance with institutional guidelines and the German animal protection legislation. Seventeen male Wistar rats (290 to 350 grams; Harlan Winkelmann, Borchen, Germany) were randomly subjected to different treatment groups: (a) ischemia through MCAO for 90 minutes, followed by reperfusion (IR) after 90 minutes (n=8) compared to (b) sham-operation (n=5). These were supplemented by the groups (c) permanent ischemia through MCAO occlusion (IO) (n=3) and (d)

untreated control animal (n=1). The right external carotid artery was ligated and transsected, a 4-0 silicone–coated nylon suture was inserted through the external carotid artery stump and advanced into the internal carotid artery beyond the carotid bifurcation until reaching the anterior cerebral artery, so that blood flow to the MCA was blocked. Reperfusion was induced in IR-animals by removing the suture 90 minutes after MCAO. Neurological evaluation and scoring were performed at 0, 4 and 24 h as described elsewhere (Gerriets et al., 2004 b). After 24 hours, animals underwent MRI (Bruker PharmaScan 7.0T, 16 cm) under isoflurane anesthesia to determine ischemic lesion size. T2-relaxation-time as a marker for brain water content and lesion-related midline shift were measured to quantify vasogenic edema formation. Computer-aided planimetric assessment of the lesion and hemispheric volumes were performed using image analysis software Image J (National Institutes of Health, NIH), regions of interest in qT2 were defined in the ischemic lesions with PharmaScan software 5.1 (Bruker). After euthanasia, brains were snap-frozen and analyzed.

7.4 Apoptosis detection

Apoptosis was quantified in cultivated cells by annexin V-FITC/propidium iodide (PI) (Immunotech) flow cytometry of 20000 cells/measurement (FACSort, BD Biosciences), as previously described (Hörmann et al., 2011). Apoptotic cells were considered annexin V+/PI- signals within pre-defined gating criteria.

In rat brain, apoptosis was detected by in situ-ligation assay (ISL) as described previously (Schoppet et al., 2004). It demonstrates the apoptosis-specific form of DNA fragmentation, i.e. 3' single-base overhang, double-stranded DNA breaks. Briefly, a specific DNA probe was prepared by PCR and purified. Brain transverse

cryostate sections (6µm) were acetone-fixed, treated with proteinase K 20 µg/mL, then incubated with the digoxigenin (DIG)-labelled DNA fragment and DNA T4 ligase (Sigma) in a humidified chamber for 1 h at room temperature. For negative control, the DIG-labelled DNA fragment was omitted, for positive control, one slide was pretreated with DNAse I. Probe binding was detected by sheep anti-DIG Fab-antibody conjugated to alkaline phosphatase (Roche, Mannheim, Germany, 1:500). Color development was performed with NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) and 1 mM levamisole (Sigma) for 70 min.

7.5 Western Blot

Protein was isolated with AllPrep RNA/protein kit (Qiagen) for cells or RIPA buffer (50mM Tris pH 8,0 containing 150mM NaCl, 1% NP-40, 0,5% sodium deoxycholat, 0,1% SDS, protease inhibitor, Complete, Roche) for tissue lysates. Protein concentration was determined with bicinchoninic acid reagent (Thermo-Scientific) at 562nm. Electrophoresis and blotting was performed as described before (Hörmann et al., 2011). Primary antibodies employed were rabbit anti human NP-1 (monoclonal, Epitomics, 1:1000) and goat-anti-human PARP (R&D-Systems, 0.4µg/ml). All blots were normalized to β-actin as loading control stained with mouse-anti-β-actin (clone AC-15, Sigma, 1:20.000). Signal transduction molecules were detected with rabbit antibodies against Bcl-2, Akt, phospho-Akt (Ser473/Thr308), SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), ERK1/2, phospho-ERK1/2 (Thr202/204), p38 MAPK, and phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling, 1:500). Western blots were evaluated by automated densitometry using the gel analyzing function of Image J software (NIH), as previously described (Gassmann et al., 2009). In Bcl-2 blots, only one specific band was detected and evaluated. In NP-1 blots, the 130 kDa band

representing the membrane-bound receptor was evaluated, other bands at 90 and 75 kDa representing soluble NP-1 without receptor function (Gagnon et al., 2000; Cackowski et al., 2004) found in individual IR and IO animals were excluded from analysis. In PARP blots, the band at 116 kDa corresponding to intact PARP was evaluated, other bands at 89 and 24 kDa corresponding to inactive PARP cleavage products (Duriez and Shah, 1997) that are found in apoptotic specimens were excluded, as previously described (Hörmann et al., 2011).

7.6 Real-time RT-PCR

PARP-1 mRNA was quantified by real-time polymerase chain reaction (PCR) using Rotorgene (Corbett). mRNA was isolated with AllPrep RNA/protein kit (Qiagen) or TriFast reagent (Peqlab) and the concentration calculated from OD_{260}. After DNAse I (Invitrogen) treatment and inactivation, reverse transcription (RT) was performed with Omniscript RT kit (Qiagen). PARP-1 real-time PCR was conducted as recently described (Hörmann et al., 2011).

PARP-1 copy numbers, calculated from threshold cycle (C_T) values, were determined by means of serially diluted external standards that were synthesized by PCR and subsequent purification (Al-Fakhri et al., 2003 b). Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

7.7 Immunocyto- and immunohistochemistry

HUVEC grown on vitronectin-coated (2µg/ml) glass cover slips were incubated in the presence or absence of VEGF-A(165) 10ng/ml for 2d. Cells were fixed with 4% formaldehyde/PBS, incubated with Image-iT FX-signal enhancer (Invitrogen), then with mouse-anti-human VEGFR-2 (Santa Cruz, clone Y-23, 1µg/ml) and rabbit-anti-

human NP-1 (5µg/ml) antibodies 16h at 4°C, thereafter with Alexa Fluor 488- and Cy3-labeled secondary antibodies 1h and DAPI 2µg/ml 20min, finally covered with ProLong Gold antifade reagent (all Invitrogen). Negative controls were conducted with an irrelevant primary antibody or PBS. Immunofluorescence was analyzed by means of an AxioVision microscope with optical sectioning using ApoTome.2 structured illumination technology (Zeiss).

Transverse cryostat sections (6µm) were acetone-fixed, incubated with 5% normal serum/PBS, then with primary antibodies: goat anti-PARP (R&D-Systems, 1:20), goat anti-VEGF (R&D-Systems, 1:50), mouse anti-VEGFR-2 (Santa Cruz, clone Y-23, 1µg/ml), rabbit anti-NP-1 (Invitrogen, 5µg/ml), rabbit anti-cleaved caspase-3 (R&D-Systems,1:1000), goat anti-catalase (R&D Systems, 0,5µg/ml) or rabbit anti-superoxide dismutase-1 (SOD-1) (Santa Cruz, FL-154, 10µg/ml) as markers of oxidative stress, rabbit anti-von-Willebrand-factor (Dako, 1:200) for 2-16h at 4°C. Subsequently, slides were incubated with biotinylated secondary antibodies, streptavidin-alkaline phosphatase-conjugate, Fast Red, and Mayer's haemalum counterstaining (all Sigma). Negative controls were conducted with an irrelevant primary antibody or PBS. Immunostaining was semiquantitatively evaluated by two investigators.

7.8 Statistics

For all analyses, 3 to 7 independent experiments were performed in triplicate (PCR) or duplicate (all other experiments) and results were expressed as mean +/-SD. Histological specimens were evaluated semi-quantitatively by light microscopy at 200-fold magnification through two experienced investigators independently: Immunohistochemical data were analyzed by counting the number of positive cells

(with a specific, bright-red staining) and identifying the cell type, the apoptotic index by counting the number of ISL positive (dark-blue) nuclei, both in four fields of view at 200-fold magnification per hemisphere and calculating the mean percentage +/-SD, respectively. In unpaired, two-tailed Student's t-test, $p<0.05$ indicated significant differences, $p<0.01$ indicated highly significant differences.

8 Results

NP-1 increases apoptosis inhibition induced by VEGF/VEGFR-2 interaction

Preincubation of HUVEC or EA.hy.926 cells with VEGF-A(165) 1-100ng/ml resulted in a significant reduction of the apoptotic rate compared to non-VEGF-treated cells. VEGF-A(165) increased endothelial resistance to apoptosis induced by cRGDfV that inhibits integrin binding to the vitronectin matrix in HUVEC (Fig. 1A) and EA.hy.926 cells. The strongest increase in apoptosis resistance was demonstrated for pre-incubation with VEGF-A(165) over 6d (at 48h-intervals) compared to 24h, matching our previous results (Hörmann et al., 2011). Preincubation with VEGF-A(121) reduced EC apoptosis induced by cRGDfV significantly less than VEGF-A(165), yet increased the resistance to apoptosis (Fig. 1A). VEGF-A(121) binds predominantly to VEGFR-2, but has neglectable binding affinity to NP-1, whereas VEGF-A(165) binds both receptors (Zachary, 2001). Incubation with VEGF-A(165) alone had no influence on the apoptotic rate, whereas VEGF-A(121) slightly increased apoptosis compared to the negative control (Fig. 1A). Incubations with receptor inhibitors SU5416 10µM and/or A7R 10µM, selective antagonists of VEGFR-2 or NP-1, respectively, revealed that blocking NP-1 had no significant influence on the induction of apoptosis resistance through VEGF-A(165) in the presence of cRGDfV. However, simultaneous

inhibition of VEGFR-2 and NP-1 or blocking VEGFR-2 alone, respectively, abolished the apoptosis inhibiting effect of VEGF (Fig. 1B). Therefore, NP-1 signaling alone in the presence of VEGFR-2 inhibitor could not prevent apoptosis induction indicating a role for NP-1 as the coreceptor of VEGFR-2. Negative controls (Fig. 1B), A7R inhibitor controls or incubations only with VEGF-A(165) (data not shown) displayed a low apoptotic rate. Incubation with SU5416 alone or A7R together with SU5416 with or without VEGF showed a small, but non-significant amount of apoptosis induction compared to the negative control (Fig. 1B). Taken together, NP-1 augmented the anti-apoptotic VEGF effect conveyed through VEGF/VEGFR-2 interaction as demonstrated by incubations with VEGF isoforms, but importantly, NP-1 did not exert an independent apoptosis-inhibiting effect as shown by inhibitor experiments.

NP-1 augments the induction of PARP expression by VEGF

In HUVEC, incubation with VEGF-A(165) 1-100ng/ml over 6d at 48h-intervals caused a significantly higher increase in intact PARP protein (116kD), maximum 2.2-fold, compared to incubation with VEGF-A(121) 1-100ng/ml, maximum 1.8-fold, as demonstrated by Western blot densitometry normalized to β-actin (Fig. 2A). Real-time RT-PCR revealed a significant, 3.7-fold increase in PARP-1 mRNA production normalized to the house keeping gene GAPDH mRNA after incubation with VEGF-A(165) for 16h compared to negative control preceding the increase in PARP protein. VEGF-A(121) induced a 2.8-fold increase in PARP mRNA that was smaller than the VEGF-A(165) effect (Fig. 2A). Therefore, binding of both receptors by VEGF-A(165) induced a stronger PARP response than binding of predominantly VEGFR-2 by VEGF-A(121). This is indicative of NP-1's role as a VEGFR-2 coreceptor.

Incubation with VEGF-A(165) and the VEGFR-2 inhibitor SU5416 showed a significant reduction of PARP protein expression compared to incubation with VEGF-A(165) alone (Fig. 2B). Coincubation of VEGF-A(165) with the NP-1 inhibitor A7R showed a comparable increase in PARP protein compared to control as incubation with VEGF alone indicating that NP-1 inhibition (in the absence of VEGFR-2 inhibition) had no effect on the regulation of PARP by VEGF. In contrast, combined incubation of VEGF-A(165) with A7R and SU5416 further reduced PARP protein compared to incubation with VEGF and SU5416 (Fig. 2B). Incubations with A7R and/or SU5416 alone (inhibitor controls) had no effect on PARP protein expression (Fig. 2B). The increase in PARP-1 mRNA production (normalized to GAPDH mRNA) through incubation with VEGF-A(165) was significantly reduced by coincubation of VEGF with SU5416 or with A7R and SU5416, respectively. Incubation with VEGF and A7R lead to a small, yet significant reduction of PARP mRNA compared to VEGF incubation. Inhibitor controls showed no influence on PARP mRNA production (Fig. 2B). Thus, NP-1 was demonstrated to augment VEGF-dependent induction of PARP expression as a coreceptor to VEGFR-2. These results were reproduced in the EC line EA.hy.926.

VEGF has previously been described to induce the intracellular apoptosis inhibitor Bcl-2 thereby reducing endothelial apoptosis (Gerber et al., 1998 a). In the present study, incubation with VEGF-A(165) 10ng/ml produced a 1.2-fold increase in Bcl-2 protein that did not reach statistical significance. This increase was smaller than the significant elevation in PARP expression induced by VEGF-A(165) (Fig. 2C). This finding underlined the relevance of the VEGF effect on PARP expression as compared to Bcl-2 expression in the context of apoptosis inhibition.

VEGF-dependent signal transduction leading to the induction of PARP expression was found to be mediated by phosphorylation of Akt and JNK, as demonstrated by Western blot of phosphorylated kinase correlated to total kinase. MAPK p38 and ERK1/2 did not show phosphorylation following stimulation with VEGF-A(165) (Fig. 2D). These results were supported by experiments with Akt inhibitors triciribine and Akti-1/2 as well as SAPK/JNK inhibitor SP600125 incubated with VEGF-A(165). Therefore, VEGF-A(165) signaling via VEGFR-2 and NP-1 is conveyed by Akt and JNK activation.

NP-1 expression colocalizes with VEGFR-2 upon VEGF stimulation and is abolished by apoptosis

VEGFR-2 and NP-1 were expressed on the cell membrane and uniformly distributed in the cytoplasm of HUVEC cultivated without VEGF supplement (Fig. 3). The receptors showed colocalization at several sites of the cell surface, demonstrated by immunofluorescence using 3D-scanning technique of an optical sectioning microscopic device. Upon stimulation with VEGF, NP-1 colocalized extensively with VEGFR-2, the receptors clustered on the cell surface and in the perinuclear space, supporting the VEGFR-2 coreceptor function of NP-1 (Fig. 3). Interestingly, in EC undergoing apoptosis induced by adhesion inhibition with cRGDfV, immuno-detectable NP-1 was dramatically reduced and VEGFR-2 distribution was changed to a disseminated, less intense staining pattern. Only few perinuclear receptor clusters containing colocalized NP-1 and VEGFR-2, probably rests of endocytosed receptor clusters, could be demonstrated in cells undergoing apoptosis (Fig. 3). Results in EA.hy.926 cells were comparable. This indicated an organizational and functional loss of VEGF receptor integrity in cells undergoing apoptosis.

Cerebral ischemia is associated with increased NP-1 and PARP expression and related to endothelial apoptosis

The in-vitro data were corroborated by a permanent (IO) and a temporary (IR) rat cerebral ischemia model that were characterized by endothelial apoptosis mainly in the ischemic hemisphere, as identified by ISL assay. Both models showed vasogenic brain edema on the ischemic side, accompanied by tissue destruction that was more prominent the IO model. The edematous lesions corresponded to the lesions found on MRI. In the IR model that underwent reperfusion after 90 minutes, the number of apoptotic endothelial and neuronal cells was significantly increased in the ischemic hemisphere as compared to either the contralateral hemisphere or sham-operated and untreated controls (Fig. 4A). Individual apoptotic nuclei were detected in the contralateral hemisphere in the vicinity of the cerebral midline, possibly as a consequence of the space occupying effect due to vasogenic edema formation. Comparable results were demonstrated for IO, however, IR showed a higher rate of apoptotic cells than IO (Fig. 4A), possibly due to the excessive tissue destruction in the ischemic brain of the IO model that reduced the number of intact vessel and neurons.

Neurological evaluation and scoring performed at 0, 4 and 24 h as well as cerebral MRI after 24 h, evaluating T2-relaxation-time and midline shift, revealed clinical and radiological signs of cerebral ischemia with ensuing vasogenic edema formation and cerebral infarction in IR or IO exposed animals. The mean ischemic lesion volume, as determined by MRI at time point 24 h, was 31.5% ± 18.7% in the IO group and 37.0% ± 10.1% in the IR group with no statistical difference between the groups

(p>0.05). The values were significantly different from sham- and non-operated control animals (0.0% ± 0.0%). Clinical scores after MCAO of the IO and IR groups were significantly different from sham- and non-operated animals (p<0.01).

Brain sections analyzed by immunohistochemistry showed increased expression of NP-1 and VEGFR-2 in the endothelium and of NP-1 in neurons of the ischemic hemisphere in IO and IR rat models (Fig. 4B). In the IR model, NP-1 expression of neurons and the endothelium was higher on the ischemic than on the contralateral side (Fig. 4B), however, only the number of NP-1 positive vessels was significantly different (Fig. 4C). In IO and IR, we generally observed a significantly higher vascular NP-1 expression in regions of endothelial apoptosis compared to brains of non-operated and sham-operated animals. VEGFR-2 showed a similar expression pattern as NP-1 in vascular structures in the IO and IR model with an increased VEGFR-2 expression on the ischemic compared to the contralateral side, but no expression in neurons (Fig. 4B). PARP immunostaining was increased in both hemispheres in the endothelium as well as in neurons compared to sham-operated and non-operated animals (Fig. 4B). In the IR model, PARP immunostaining was significantly higher in the neurons on the ischemic side than on the contralateral side; for EC, there was no difference between the hemispheres (Fig. 4C). However, the PARP antibody detects intact PARP and its degradation products so that an increased PARP immunoreactivity might also be due to neuronal apoptosis induction in the ischemic region. VEGF expression of pial and glial cells as well as individual neurons in the ischemic hemisphere and weaker in the contralateral hemisphere was demonstrated. In the ischemic hemisphere, increased immunostaining of activated (cleaved)

caspase-3, catalase (Fig. 4B) and SOD-1 expression was found. Von Willebrand factor staining demonstrated the integrity of the endothelial lining (Fig. 4B).

Whole tissue lysates separately prepared from both hemispheres of every individual animal showed an increased NP-1 protein expression in the IR model compared to the controls as demonstrated by densitometry of Western blots. NP-1 expression was higher in the contralateral hemisphere of the IR model compared to the ischemic hemisphere (Fig. 4D). However, the tissue lysates consisted of whole brain tissue and did not allow for differentiation of cell types. PARP protein levels were almost identical on both sides and remained unaltered compared to the control animals ($p>0.05$) (Fig. 4D). Note that an unchanged PARP expression under the pro-apoptotic conditions of ischemia indicates a net increase in PARP expression, since PARP is degraded immediately during apoptosis induction. In summary, NP-1 together with VEGFR-2 and PARP are increased in cerebral ischemia in the context of imminent vascular and neuronal apoptosis.

9 Discussion

Vascular apoptosis is a consequence of cerebrovascular ischemia, but in contrast to neuronal apoptosis, vascular apoptosis in the brain has not been intensively investigated. However, protective therapeutic strategies for the cerebrovascular system warrant the characterization of vascular apoptosis inhibition to understand the regulation of endothelial integrity (Broughton et al., 2009). This study investigated mechanisms underlying cell survival in the vasculature in cerebrovascular ischemia.

Occlusion of cerebral vessels first leads to increased endothelial permeability and temporary impairment of the blood-brain-barrier (Dirnagl et al., 1999), induced by

VEGF as an early response to hypoxia (Schoch et al., 2002). VEGF has both, beneficial and negative effects on the blood-brain-barrier: Next to inducing hyperpermeability, it is the strongest anti-apoptotic factor for vascular EC (Zachary, 2001) and promotes endothelial migration and proliferation (Kowanetz and Ferrara, 2006; Ferrara, 2009). However, VEGF-induced hyperpermeability can be adapted quickly through the involvement of vesiculo-vacuolar organelles that regulate intracellular fluid and molecule transport (Teesalu et al., 2009). When the cerebral blood flow is restored within a short time period, the changes in the endothelial barrier are reversed. Longer lasting interruptions in perfusion, i. e. 90 minutes of ischemia followed by reperfusion, lead to apoptosis of the vascular endothelium (Li et al., 1995). This encompasses the breakdown of the blood-brain-barrier with consequent cerebral edema and neuronal damage (Dirnagl et al., 1999; Gerriets et al., 2004 a). Mechanisms inhibiting endothelial apoptosis therefore not only increase endothelial resistance to pro-apoptotic stimuli, but also support vascular integrity and may partly or completely prevent breakdown of the blood-brain-barrier in situations of vascular stress (Fisher, 2008).

Endothelial apoptosis in cerebral ischemia is induced by the extrinsic apoptosis pathway with caspase-8 and caspase-3 activation (Zhou et al., 2004). One of the mechanisms by which endothelial apoptosis is prevented physiologically is an increase in apoptosis resistance induced by VEGF that counteracts the caspase pathway by regulating PARP expression (Hörmann et al., 2011). In the present study, apoptosis was induced by the αv-integrin-specific adhesion inhibitor cRGDfV as a well documented approach to apoptosis induction through caspase-3 activation (Al-Fakhri et al., 2003 a) that in turn cleaves and inactivates PARP (Virag, 2005). PARP

is a DNA-repair enzyme counteracting pro-apoptotic stimuli, but it is also a cofactor to transcription factors associated with inflammation like NF-κB and STATs (Aguilar-Quesada et al., 2007). The exact mechanisms by which PARP counteracts apoptosis have not been elucidated, whether by its DNA-regenerating function or by the regulation of gene expression (Virag, 2005). However, PARP is a two-faced molecule that can also cause necrosis when excessive cellular stress occurs (Aguilar-Quesada et al., 2007). PARP has also been attributed a role in inflammatory responses. Which of its functions PARP exerts depends on the cell's actual activation state (Virag, 2005; Aguilar-Quesada et al., 2007).

Since VEGF can exert these different effects on the vascular system, its function needs to be fine-regulated to adapt to situative responses. A possible candidate molecule could be NP-1, a transmembrane, non-tyrosinkinase VEGF receptor, that influences the interaction of VEGF with its main receptor, the tyrosinkinase receptor VEGFR-2 (Soker et al., 1998; Soker et al., 2002). NP-1 was first described as a semaphorin receptor and neuronal cell guidance molecule (Fujisawa and Kitsukawa, 1998) and was subsequently identified to be a VEGFR-2 co-receptor in VEGF-A(165) binding during angiogenesis (Soker et al., 1998; Soker et al., 2002). However, individual reports have attributed an independent receptor function to NP-1 on EC (Wang et al., 2007; others reviewed in Ylä-Herttuala et al., 2007) and on fibroblasts (Kim et al., 2006). NP-1 is also expressed on different non-vascular cells like neurons, T-cells and dendritic cells (Lepelletier et al., 2007; Gu et al., 2003) and plays a role in cancer cell migration (Jia et al., 2010). NP-1 transgene and gene deletion mouse models indicated its essential role in angiogenesis, capillary formation, and in the development of the heart, aorta and large blood vessels (Kitsukawa et al., 1997; Kawasaki et

al., 1999). In the brain of NP-1 overexpressing mouse embryos, excess and dilated vessel formation was identified indicating a role for NP-1 in brain EC migration, proliferation, survival, and angiogenesis (Kitsukawa et al., 1995).

The role of NP-1 in disease has predominantly been investigated in tumor angiogenesis (Liu et al., 2002; Eccles and Welch, 2007), but its role as a VEGF receptor in (cerebro-)vascular pathologies is largely unknown. NP-1 expression has been related to neovascularization and angiogenesis in the longer-term course of ischemic lesion remodeling in the rat or mouse model (Zhang et al., 2001; Beck et al., 2002), but otherwise no evidence exists on the role of NP-1 in acute cerebrovascular disease. We hypothesized that NP-1 represents the fine-regulating element in the VEGF/VEGF receptor system modulating the VEGF response of the endothelium. In this study, we found evidence for the enhancement of important effects of VEGF by NP-1. VEGF-dependent apoptosis inhibition and induction of PARP expression transduced by VEGFR-2 were augmented by NP-1. Endothelial NP-1 and VEGFR-2 expression was found to be increased in cerebral ischemia in relation to apoptosis and PARP expression.

NP-1 was characterized as a coreceptor to VEGFR-2 in VEGF-A(165)-dependent apoptosis inhibition and upregulation of PARP expression: VEGF-A(121) that binds predominantly to VEGFR-2 (Soker et al., 1998) reduced apoptosis significantly less than VEGF-A(165) that binds to VEGFR-2 and NP-1. VEGF-A(165) was first described as the only VEGF form that binds to NP-1 on EC (Soker et al., 1998), lateron binding of VEGF-A(121) to NP-1 was described although without binding enhancement of VEGF-A to VEGFR-2 (Whitaker et al., 2001; Pan et al., 2007).

VEGF-A(121) actually lacks the NP-1 binding site in exon 7 found in VEGF-A(165) (Zachary, 2001), but VEGF-A(121) might bind to NP-1 via the C-terminal region (Pan et al., 2007). In this study, incubations with VEGFR-2 and NP-1 inhibitors supported the VEGFR-2 coreceptor function of NP-1 for apoptosis inhibition as well as for PARP expression. VEGF signaling via both receptors was transduced by Akt and JNK activation as shown by detection of kinase phosphorylation and by Akt and JNK inhibitor experiments.

Close functional association of NP-1 and VEGFR-2 on EC (Soker et al., 1998; Giraudo et al., 1998; Petrova et al., 1999) is reflected in the colocalization of both receptors that we demonstrated particularly after VEGF stimulation. The VEGF-induced clustering of VEGFR-2 and NP-1 in the perinuclear space may originate from endocytosed receptor complexes, since NP-1 is internalized together with VEGFR-2 upon VEGF-A(165) stimulation forming a functional receptor complex localized to endocytic vesicles containing Rab11 (Ballmer-Hofer et al., 2011). Additionally, intracellularly localized NP-1 may be associated with vesiculo-vacuolar organelles that are responsible for VEGF-induced transcellular transport (Teesalu et al., 2009). Unexpectedly, the expression pattern of the VEGF receptors changed dramatically in cells undergoing programmed cell death with an almost complete disappearance of immunodetectable NP-1 and a disseminated, weak VEGFR-2 expression. The disassembly of VEGF receptors during apoptosis may point to a loss of VEGF responsiveness of EC, since signal enhancement by NP-1 is abolished. The NP-1 decrease may be caused by receptor internalization and degradation or alternatively by receptor shedding: ADAM-10 has been described to shed NP-1 from EC releasing soluble NP-1 into the circulation, whereas ADAM-17 is the sheddase of VEGFR-2;

NP-1 shedding is not inducible by VEGF in contrast to VEGFR-2 (Swendeman et al., 2008). Shedding of VEGF receptors supplies soluble NP-1 to more distant vascular sites, remarkably, soluble NP-1 acts as VEGF-antagonist by binding VEGF-A(165) (Gagnon et al., 2000). Reduction of cellular NP-1 enhances the endothelial susceptibility to apoptosis, this effect might be increased when NP-1 is released in its soluble form thereby inhibiting VEGF functions.

Bcl-2, an inhibitor of the intrinsic pathway of apoptosis, has previously been identified as a target of VEGF to reduce endothelial apoptosis (Gerber et al., 1998 a). In rheumatoid synovia, twofold upregulation of Bcl-2 by VEGF through NP-1 has also been described thereby protecting from apoptosis (Kim et al., 2006). However, since endothelial apoptosis is mainly induced via the extrinsic pathway in cerebral ischemia (Zhou et al., 2004) and in atherosclerotic vascular remodeling (Al-Fakhri et al., 2003 a), a caspase mediator like PARP is more likely to influence EC apoptosis. As a control experiment, we investigated Bcl-2 regulation by VEGF, but did not find the two- to threefold increase in Bcl-2 protein levels described previously (Gerber et al., 1998 a) in our experimental settings. Instead, the effect of VEGF on the expression of Bcl-2 was smaller than that on PARP. Upregulation of PARP was therefore shown to be the more prominent effect of VEGF in EC.

In the rat MCAO model, we found that upregulation of endothelial NP-1 and VEGFR-2 occurred in the short-term course of cerebral ischemia within 24 hours after occlusion or occlusion-reperfusion in the vicinity of cerebral infarcts. These regions were characterized by endothelial apoptosis and detection of cleaved caspase-3. Surprisingly, NP-1 and VEGFR-2 were upregulated not only in the vessels of the

infarct-side, but also of the contralateral hemisphere. Overall hemispheral expression analyzed from tissue lysates indicated that the expression of NP-1 protein seemed to be significantly higher on the contralateral side, possibly because of a higher number of intact vessels and neurons, since the ipsilateral hemisphere contained the infarct area. However, in immunohistochemistry, a slightly, but significantly higher number of NP-1 expressing EC was demonstrated on the ischemic side. In the close vicinity of apoptotic vessels, endothelial NP-1 and VEGFR-2 expression was highly increased.

For the first time, this study related imminent endothelial apoptosis in vessels to an increased expression of NP-1 and VEGFR-2. Previous studies on cerebrovascular NP-1 expression had mainly looked into NP-1 changes related to angiogenesis during post-ischemic brain remodeling in longer-term animal models: Upregulation of NP-1 was demonstrated in neovascularization from 7 to 28 days after focal cerebral ischemia in the rat MCAO model with permanent occlusion (Zhang et al., 2001). Vascular NP-1 was also described to be induced in the peri-infarct area related to angiogenesis during post-ischemic brain remodeling 3 to 7 days after MCAO in the mouse model; neuronal and glial NP-1 was increased as well (Beck et al., 2002).
However, the VEGF/VEGF receptor system is able to respond acutely to vascular impairment by upregulation of VEGF expression in the brain: After transient MCAO in rats, VEGF expression increases in neurons and pial cells within the first 24 hours after reperfusion (Hayashi et al., 1997). VEGF mRNA is upregulated by microglia and macrophages in the ischemic area within the first hours after MCAO (Plate et al., 1999). Both VEGF isoforms, VEGF-A(165) and -A(121), are detected after chronic or acute cerebral hypoperfusion, with a predominance of the NP-1-binding isoform VEGF-A(165) (or VEGF-A(164) in the rat) (Hai et al., 2003; Hayashi et al., 1997). In

the present study, endothelial VEGF immunoreactivity was demonstrated in brain vessels on the infarct and the contralateral side. This VEGF could be bound locally to VEGF receptors, as suggested for the fast increase after cerebral ischemia (Plate et al., 1999). As to VEGFR-2, previous studies in the rat model did not identify an increased endothelial expression during acute or chronic cerebral ischemia (Hai et al., 2003; Plate et al., 1999), although this does not entirely fit to the described increase in EC-bound VEGF. In contrast, in the mouse MCAO model, strong upregulation of vascular VEGFR-2 was found 3 days after ischemia (Beck et al., 2002).

Vascular apoptosis is induced by pro-apoptotic factors or by radical oxygen species (ROS) (Fisher 2008; Olmez and Ozyurt, 2012; Al-Fakhri et al., 2003 a) as a result of oxidative stress caused by cerebral ischemia. Oxidative stress reactively induces catalase and SOD-1 expression (Slemmer et al., 2008; Facchinetti et al., 1998), both of which were found here in the vessels of the ipsilateral hemisphere. Taken together, oxidative stress produced by ischemia could lead to a reactive upregulation of anti-apoptotic systems, such as the VEGF/VEGF receptor system, thereby causing the widespread increase of VEGF receptors in the brain. The increase in VEGF may be an anti-apoptotic response to brain injury, since VEGF can by itself induce vascular expression of NP-1 via VEGFR-2 (Oh et al., 2002).

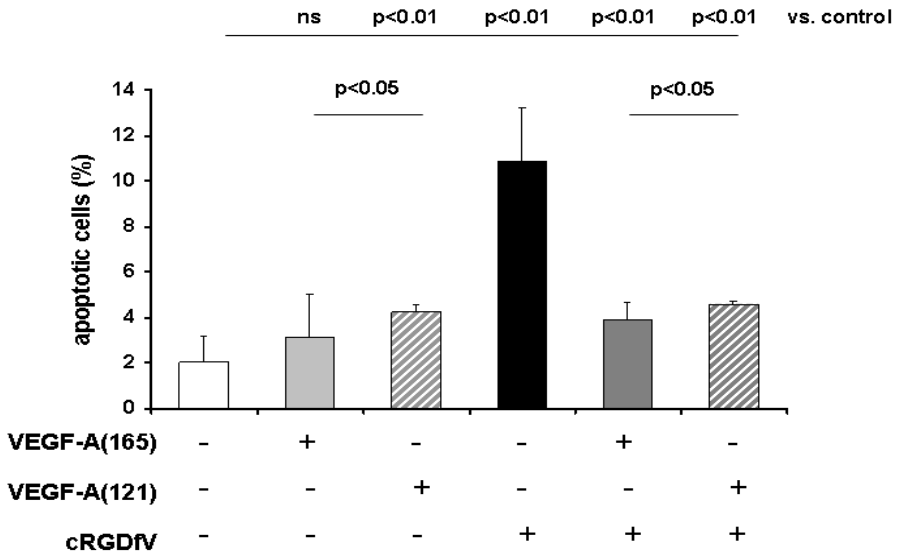
PARP immunoreactivity was increased in vessels and neurons after ischemia compared to control animals with an identical expression level in both hemispheres in whole tissue lysates. Since PARP is degraded immediately during apoptosis (Virag, 2005; Aguilar-Quesada et al., 2007), stable PARP expression in regions of apoptosis during cerebral ischemia indicated a net increase in PARP expression, possibly induced by VEGF (Hörmann et al., 2011). This may reflect a short-term, situative

response to imminent vascular and neuronal apoptosis to prevent brain damage induced by ischemia. However, overactivated PARP can also cause cellular damage, since PARP can switch from DNA repair to the enhancement of inflammation and oxidative stress (Virag, 2005; Aguilar-Quesada et al., 2007). As a result of ischemia, oxidative stress could lead to an increased risk of PARP overactivation and necrotic cell death. Induction of PARP expression by VEGF must therefore be controlled to avoid PARP overactivation. Since NP-1 modulates the VEGF-dependent upregulation of PARP, NP-1 ensures a regulated endothelial response to ischemia and apoptosis. NP-1 may therefore represent the best candidate for a key modulator ensuring the maintenance of endothelial integrity in cerebrovascular diseases.

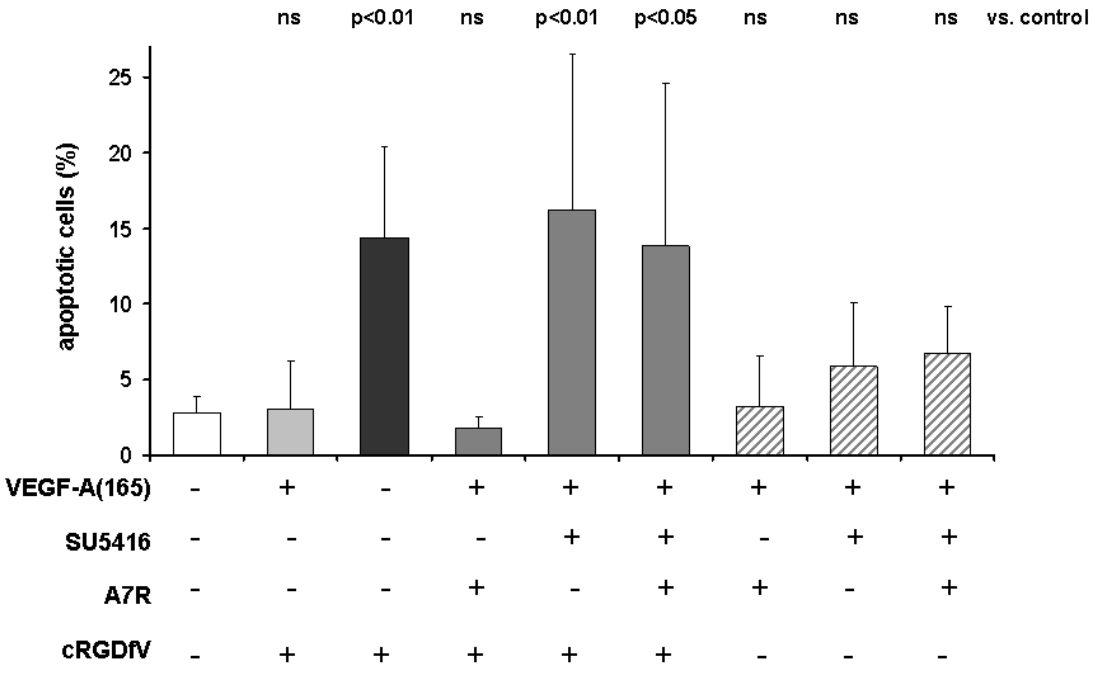
Understanding the mechanisms of apoptosis inhibition in the cerebral vasculature could ultimately lead to particular or supplemental therapies targeting the endothelium to prevent apoptosis. Modulation of the VEGF/VEGF receptor system through NP-1, targeting PARP expression and endothelial apoptosis, is a promising strategy for post-stroke treatment in the management of cerebral ischemia.

10 Figures and figure legends

Fig. 1. Endothelial resistance to apoptosis is augmented by the vascular endothelial growth factor (VEGF) receptor neuropilin-1 (NP-1).

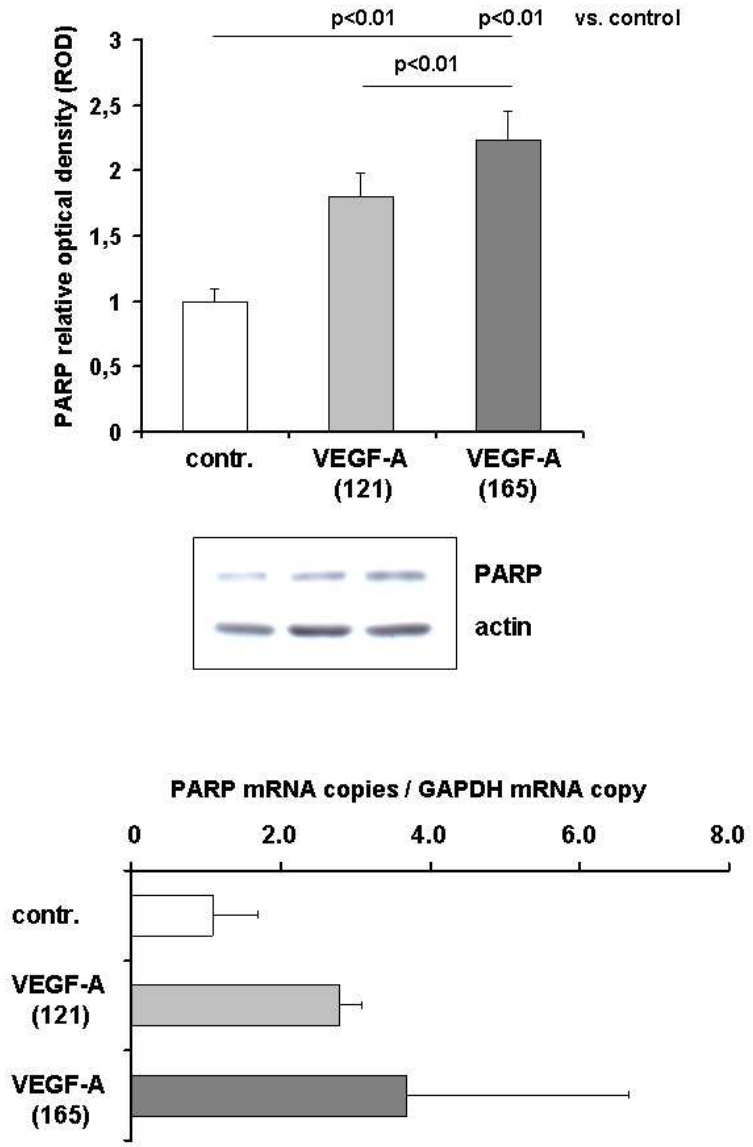


(**A**) Pre-incubation of HUVEC with VEGF-A(165) 10ng/ml (6d, 48h-intervals) significantly reduced apoptosis induced by cRGDfV 5µg/ml compared to cells not pre-incubated with VEGF. VEGF-A(121) 10ng/ml that binds predominantly to VEGF receptor-2 (VEGFR-2) showed a significantly smaller effect on apoptosis inhibition than VEGF-A(165) that binds both receptors indicating an increase of the VEGF effect through the involvement of NP-1. VEGF-(A165) alone did not influence apoptosis, but VEGF-A(121) alone lead to a small increase in apoptosis underlining the importance of NP-1 for EC survival.

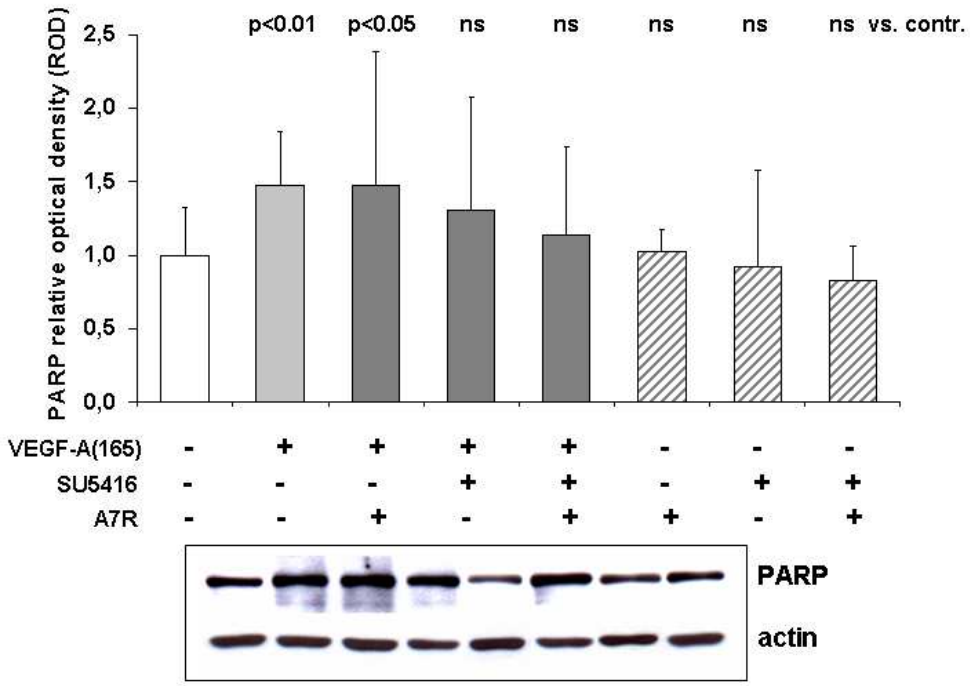


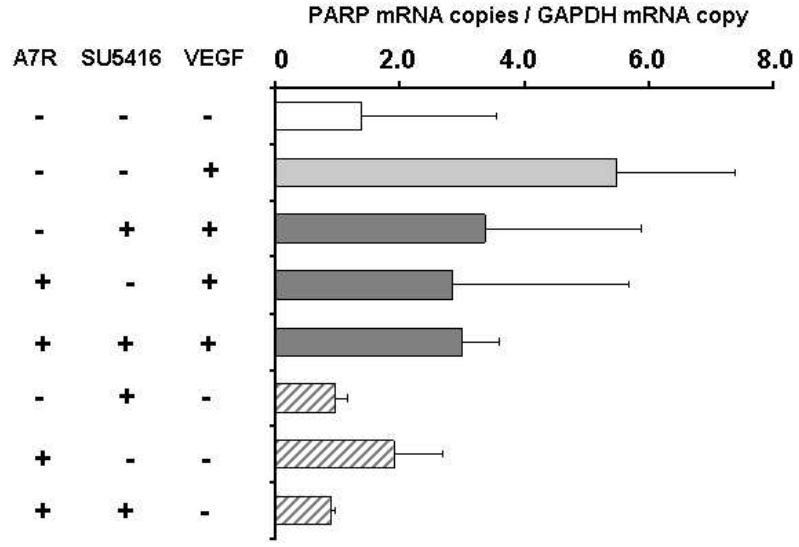
(**B**) The impact of NP-1 on apoptosis inhibition was supported by experiments with VEGFR-2 inhibitor SU5416 and NP-1 inhibitor A7R. Pre-incubation of HUVEC with VEGF-A(165) 10ng/ml over 6d inhibited cRGDfV-induced apoptosis, this was blocked by co-incubation with SU5416 10µM or the combination of SU5416 with A7R 10µM. In contrast, A7R had no effect on apoptosis inhibition in cells pre-incubated with VEGF-A(165) compared to incubation with VEGF and cRGDfV. Incubations with VEGF-A(165) and inhibitors (without cRGDfV) showed no apoptosis induction through A7R and a small, non-significant rise in the apoptotic rate through SU5416 or both inhibitors in the presence of VEGF. Control incubations with inhibitors alone showed no significant induction of apoptosis, incubations with inhibitors and cRGDfV had results similar to cRGDfV alone (data not shown). Data in (A) and (B) are mean +/- standard deviation of 4-5 independent experiments. Statistical significance of differences is indicated by a p-value of <0.05 (t-test).

Fig. 2. NP-1 increases PARP expression induced by VEGF.



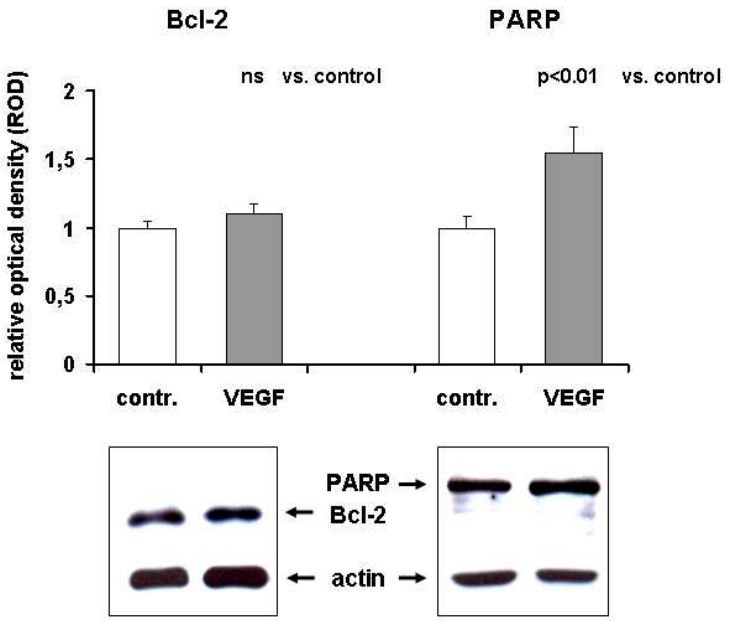
(**A**) VEGF-A(165) 10ng/ml induced a significantly stronger increase in PARP protein than VEGF-A(121) 10ng/ml demonstrated by Western blot and automated densitometry. PARP protein was normalized to β-actin as loading control. Additionally, VEGF-A(165) had a grea-ter effect on PARP mRNA production than VEGF-A(121), quantified by real-time RT-PCR and normalized to GAPDH mRNA copy. These data indicate a co-receptor role for NP-1 augmenting the effect of VEGF/VEGFR-2 interaction on PARP expression.





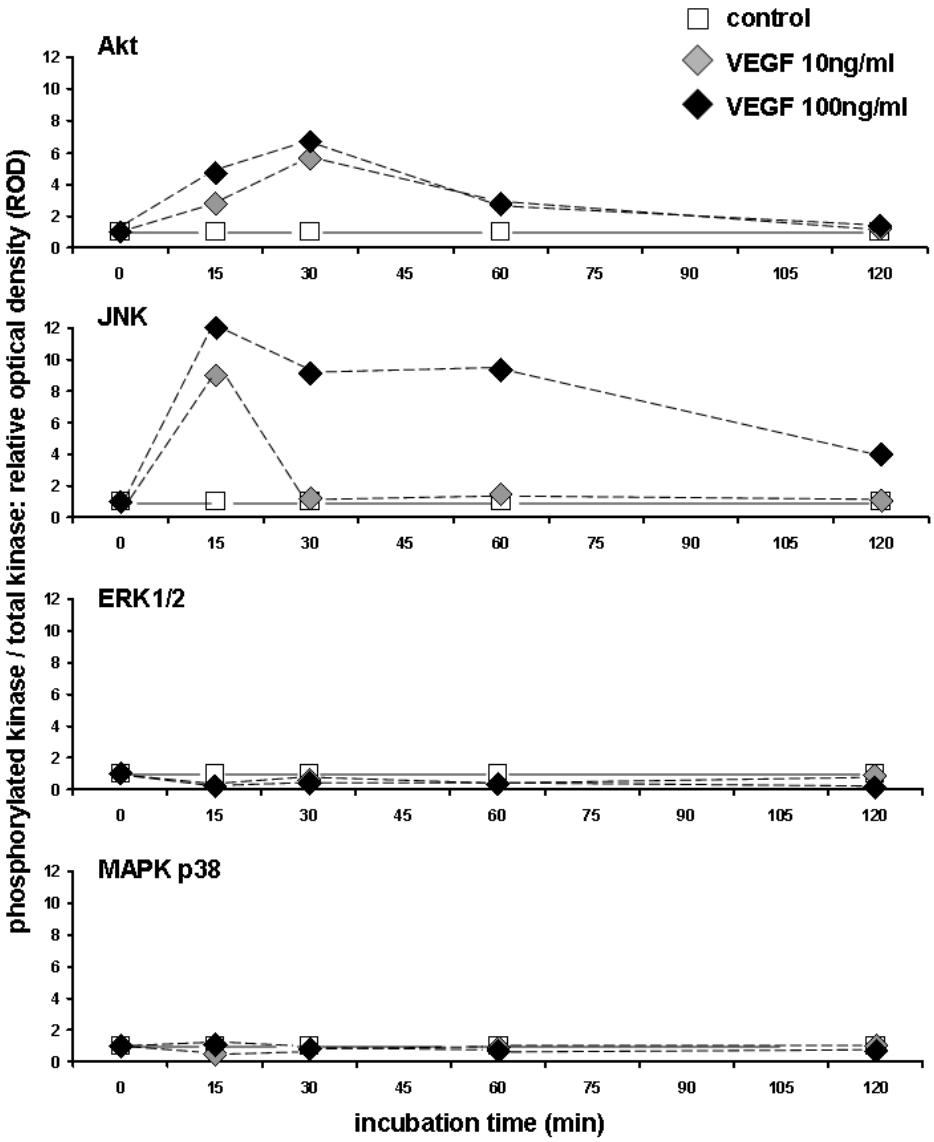
(**B**) Incubation with VEGF-A(165) and SU5416 (VEGFR-2 inhibitor) demonstrated a significantly reduced expression of PARP protein compared to incubation with A7R (NP-1 inhibitor) and VEGF or with VEGF alone. Combined incubation A7R and SU5416 with VEGF-A(165) further reduced PARP protein emphasizing the coreceptor function of NP-1 in PARP gene induction. PARP mRNA production was influenced accordingly by incubation with VEGF-A(165), SU5416 and A7R, quantified

by real-time RT-PCR and normalized to GAPDH mRNA. However, PARP mRNA production levels from incubations with VEGF-A(165) and SU5416, A7R or both inhibitors, respectively, were not statistically different. Control incubations with inhibitors only had no influence on PARP protein or mRNA expression.



(**C**) VEGF-A(165) induced the expression of the anti-apoptotic intracellular protein Bcl-2, however, the expression level was significantly smaller than that of PARP protein and did not reach statistical significance compared to negative control.

Data in (A), (B) and (C) are the means of 3 independent experiments. Representative blots of experiments are supplemented to the densitometry data.



(**D**) Analysis of signal transduction revealed activation of Akt and JNK, whereas ERK1/2 and MAPK p38 remained unphosphorylated. Short term incubations over 15-120 min were carried out with VEGF-A(165) 10-100ng/ml and analyzed by Western blot automated densitometry of the respective phosphorylated to total kinase proteins. Data are the means of 5 independent experiments.

Fig. 3. The colocalized receptors VEGFR-2 and NP-1 change their expression pattern upon VEGF stimulation and during apoptosis induction. In control incubations of HUVEC without VEGF ("contr."), VEGFR-2 and NP-1 were demonstrated on the endothelial cell membrane and in the cytoplasm with a disseminated distribution pattern and only occasional colocalization. After incubation with VEGF-A(165) 10ng/ml ("VEGF"), clustering and almost complete colocalization of VEGFR-2 and NP-1 was demonstrated by optically sectioned immunofluorescence staining (ApoTome.2, Zeiss). Clusters of colocalized receptors we detectable in the cytoplasm indicating receptor endocytosis. Apoptosis induction with cRGDfV 5µg/ml ("cRGD") dramatically reduced NP-1 expression leaving no NP-1 on the cell surface and only few clusters of NP-1 with VEGFR-2 in the perinuclear space. The VEGFR-2 expression pattern became diffusely distributed in cells undergoing apoptosis. VEGFR-2 is stained green (Alexa Fluor 488), NP-1 red (Cy-3), nuclei are stained blue (DAPI); 600-fold magnification. The scale bar represents 100 µm. A representative example of 3 independent experiments is shown. Individual sites of colocalized receptors are indicated by arrows.

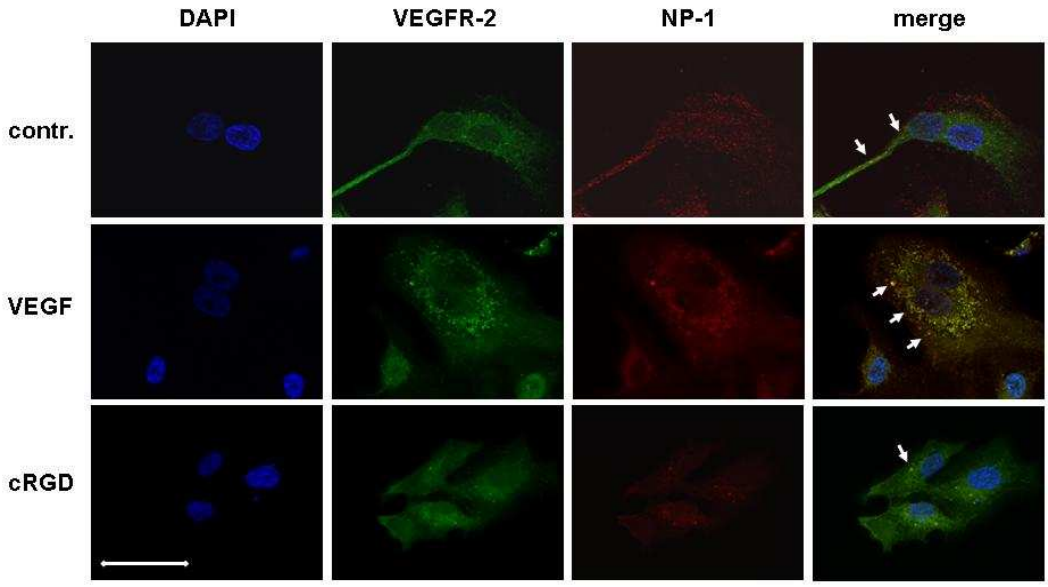
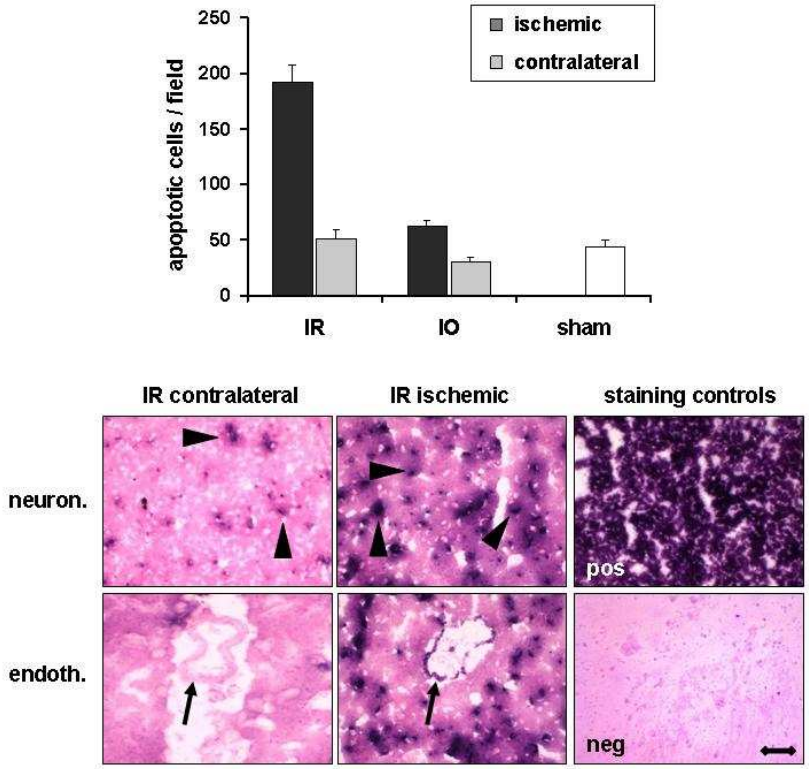
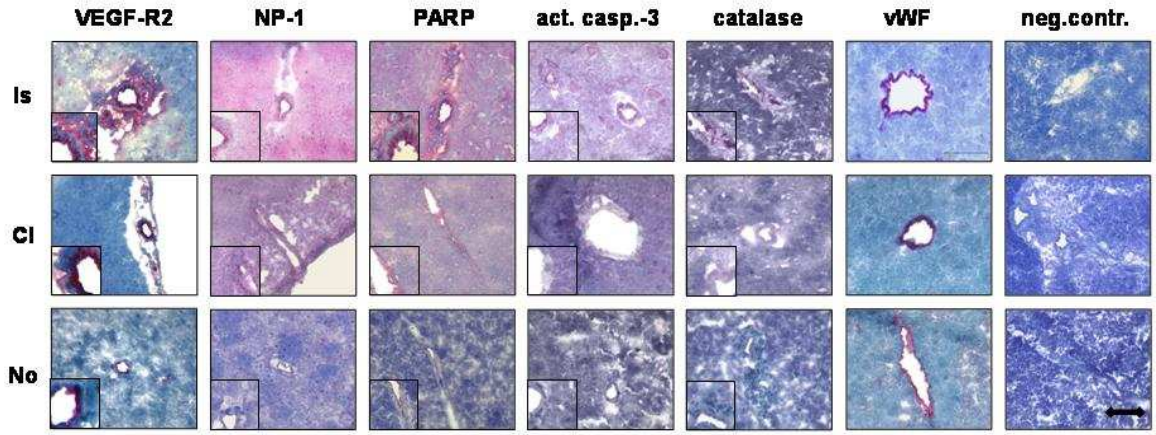


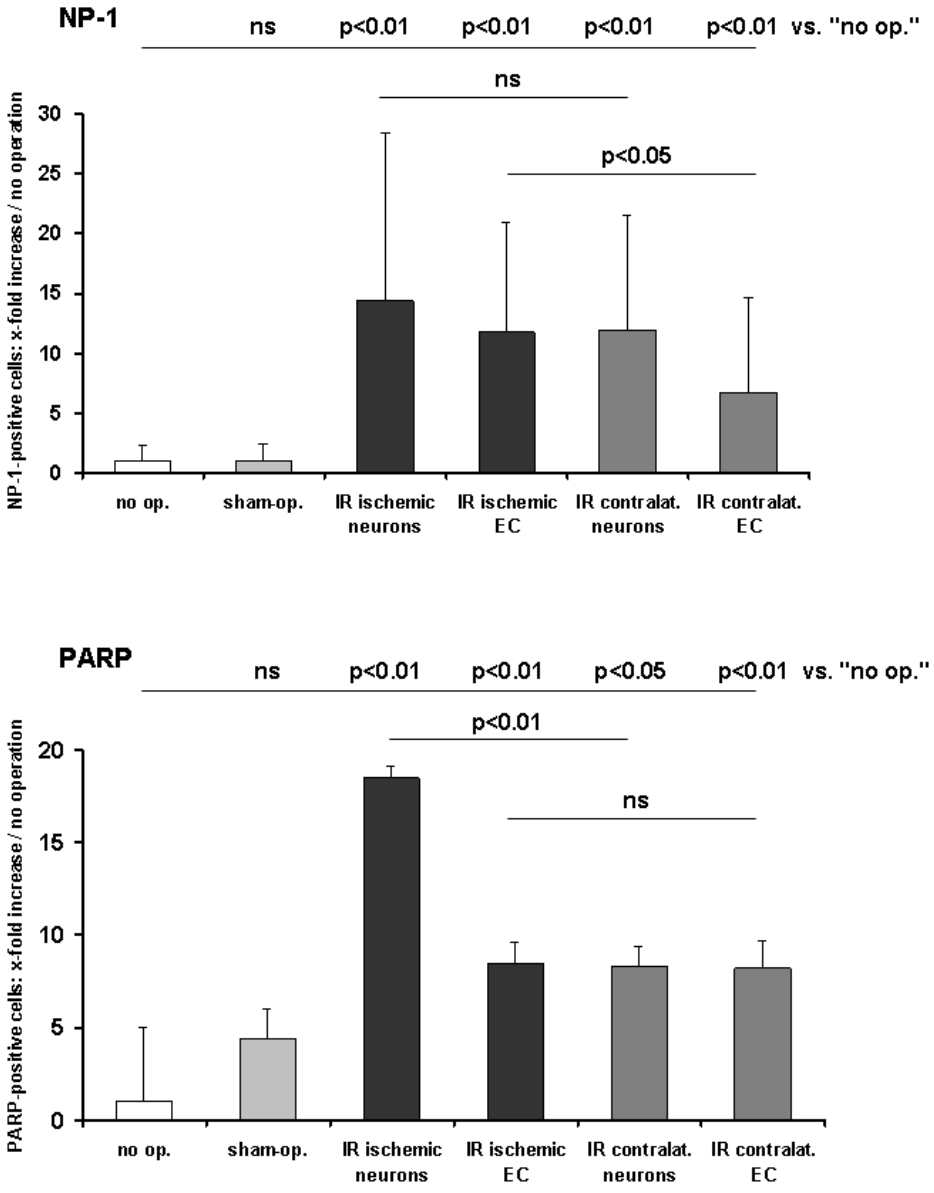
Fig. 4. Cerebral ischemia of the rat is characterized by endothelial apoptosis and changes in the expression of VEGF receptors and PARP.



(**A**) In the ischemia-occlusion (IO, n=3) and ischemia-reperfusion model (IR, n=8), apoptosis was detected by ISL assay. The number of apoptotic cells, especially of EC, was significantly higher on the ischemic side of the IR model than of the IO model compared to sham-operated (n=5) and untreated controls (n=1). The panels show the IR model stained by ISL assay, examples of neuronal tissue (neuron.) and vascular endothelium (endoth.) of the ischemic and contralateral side are given together with staining controls (pos: positive control, DNAse I pretreatment; neg: negative control, no probe). Arrowheads in the upper panels indicate ISL-positive, apoptotic neuronal nuclei, arrows in the lower panels indicate the vascular endothelium (200-fold magnification). Data in (A) and (C) are means +/- standard deviation of two experiments counted by two investigators as described in the text.

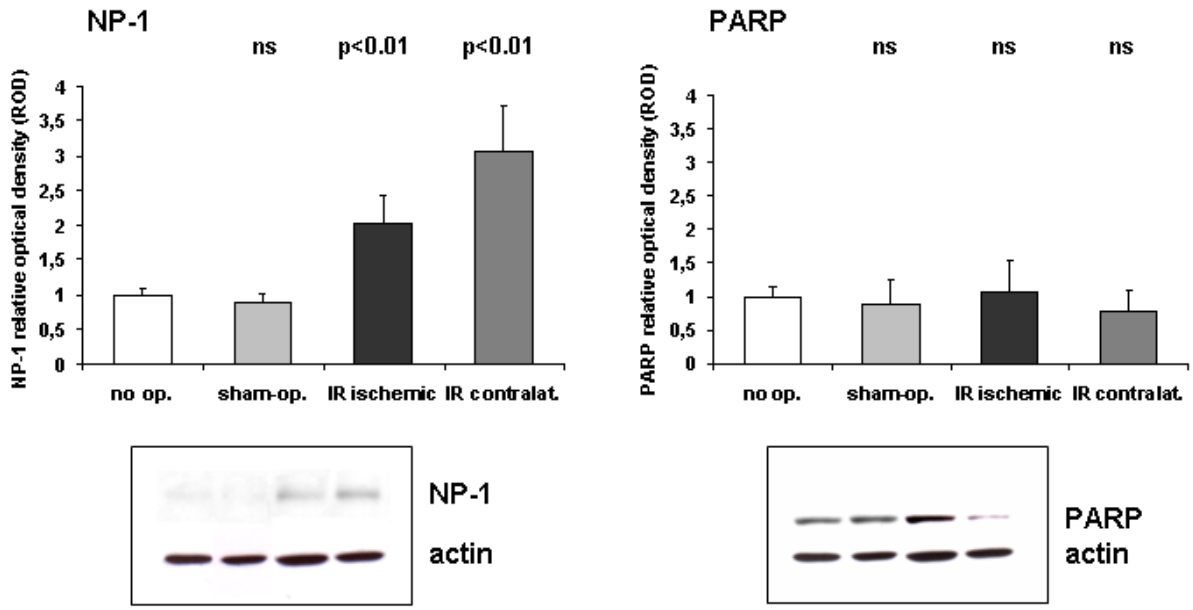


(**B**) Cryotome sections of rat brains were stained by immunohistochemistry (alkaline phosphastase-conjugate, Fast Red). Representative examples from the ischemic (Is) and contralateral (CI) hemisphere of the IR model and of an untreated, normal animal (No) are shown (200-fold magnification). The inserted panels show a close-up of the positively marked vessels at higher magnification. Increased VEGFR-2 and NP-1 immunoreactivity was detected on the endothelium of the ischemic and contralateral hemisphere in the IR model. NP-1 immunostaining was also increased in neurons in the IR model, especially on the ischemic side, compared to controls. PARP immunoreactivity was increased in the endothelium and neurons in IR animals compared to controls. Activated, cleaved caspase-3 (act. casp.-3) and catalase were increased in the vascular endothelium of the ischemic hemishpere indicating a correlation between endothelial apoptosis, increased PARP and NP-1 expression with oxidative stress. Catalase (and SOD-1, not shown) were also moderately increased in vessels of the contralateral hemisphere. As staining controls, von Willebrand factor (positive control for the endothelial lining) and the negative staining control (neg. contr.) are shown. The scale bar in (A) and (B) represents 100 µm.



(**C**) Semi-quantitative evaluation of NP-1 and PARP immunostaining revealed a highly significant increase in NP-1 and PARP positive endothelial and neuronal cells in the IR model on the ischemic (IR ischemic) and contralateral (IR contralat.) side compared to non-operated (no op.) and sham-operated (sham-op.) animals. An increased number of NP-1 and PARP positive vessels were demonstrated in the vicinity of regions of endothelial apoptosis. Comparison of the ischemic and the

contralateral hemisphere in IR animals revealed a higher number of NP-1 positive neurons and vessels on the ischemic side, but only endothelial expression was significantly different between the hemispheres. PARP positive vessels were comparably increased in both hemispheres, but a significantly higher count of PARP positive neurons was found in the ischemic compared to the non-ischemic hemisphere.



(**D**) In separately prepared whole brain tissue lysates of both hemispheres, an increased NP-1 protein expression in the IR model was found compared to controls. NP-1 expression was higher in the contralateral hemisphere than in the ischemic hemisphere demonstrated by Western blot and automated densitometry. PARP protein expression was comparably high in the IR model as in controls, no statistical difference was found between the ischemic and the contralateral hemisphere. These findings indicate a net increase in the production of PARP under pro-apoptotic conditions, since PARP is degraded during apoptosis induction. Representative blots of individual animals are supplemented to the densitometry data.

11 Funding

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